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EDITORIAL

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A CHANGE IN PUBLICATION OF MEMÓRIAS DO INSTITUTO BUTANTAN AND COLETÂNEA DE TRABALHOS DO INSTITUTO BUTANTAN.

A partir do volume 49, 1987, as "MEMÓRIAS DO INSTITUTO BUTANTAN" serão editadas em fascículos, em intervalos irregulares.

A "COLETÂNEA DE TRABALHOS DO INSTITUTO BUTANTAN" não mais será editada como volume independente, passando a ser incluída, sob a forma de resumos, no último fascículo das "MEMÓRIAS DO INSTITUTO BUTANTAN", com o título de "COLETÂNEA DE RESUMOS DE TRABALHOS PUBLICADOS PELOS PESOUISADORES DO INSTITUTO BUTANTAN".

As of volume 49, 1987, the "MEMÓRIAS DO INSTITUTO BUTANTAN" will furtheron be edited in fascicules at irregular intervals.

The "COLETÂNEA DE TRABALHOS DO INSTITUTO BUTANTAN" will no more be edited as independent volumes, but will be included in the last fascicule of the "MEMÓRIAS DO INSTITUTO BUTANTAN" as abstracts under the title "COLETÂNEA DE RESUMOS DE TRABALHOS PUBLICADOS PELOS PESOUISADORES DO INSTITUTO BUTANTAN".

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HISTORY OF THE PRIMORDIA OF SNAKE-BITE ACCIDENT SEROTHERAPY*

Oswaldo VITAL BRAZIL**

First I would like to thank the organizers of this session in honor of Vital Brazil, the founder of this Institute, for the invitation to address you on his life and scientific work. I believe this was motivated by the fact that not only I am one of his sons but also a scientist belonging to his scientific school and one of the continuators in this country of his researches on the South American snake, scorpion and spider venoms. To present, even succinctly, all of Vital Brazil's accomplishments in an exposition which must not be overly lengthy seemed to me impossible. I shall restrict myself, therefore, to present a historical summary on antivenomous serotherapy and to make, therefore, an exposition of the research that marked so deeply the destiny of this Institute, one of our country's most important and humanitarian scientific institution and certainly the most original.

The introduction of serotherapy in the treatment of snake-bite accidents was mainly due to two scientists, Albert Calmette and Vital Brazil. The former demonstrated in 1884 that the serum from animals immunized with snake venoms was capable of neutralizing them, thereby making possible its preventive and curative application to counteract their effects in the animal organism. Starting from this verification, Calmette immunized horses with snake venoms and the antivenin or "serum antivenimeux", as he called it, was distributed for use in the treatment of accidents caused by snakes in various parts of the world, in particular in Indochina, India, Australia and Europe. Vital Brazil was the first to demonstrate the specificity of the antivenins, a fact which paradoxically was neither recognized nor admitted by Calmette. Following this discovery, Vital Brazil started in 1901 to prepare mono and polyvalent antivenins — the anticrotalic, antibothropic and antiophidic sera — for use in Brazil. He was, thus, the creator of the antivenomous serotherapy on a really effective basis. His orientation — preparation of mono and polyvalent antivenins for use in a determined region — is adopted world-wide today.

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* Translation of the address given during the session in honor of Vital Brazil's memory held in 28 April 1986 at the Instituto Butantan.

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Albert Calmette
(1863 – 1933)

Calmette[4] was born in 1863 in a very old and mountainous country, the region of Auvergne, in the Massif Central of France. His ideal as a boy: to be a naval officer and to take part in the civilizing mission of his nation in distant regions. This ideal was unfulfilled for the benefit of science and humanity. A long illness contracted at the Lycée of Brest prevented him of doing so. However, he joined Brest's naval School of Medicine in 1879. After being submitted to the required examinations in 1881, he became an auxiliary physician ("aide-médecin"). Then, he was sent to serve on a war vessel in Annan and Tonkin (middle and north Vietnam). As a doctor on board, he participated in heroic first aids during the bloody naval battles between French ships and the Chinese fleet. Back to France, he submitted himself successfully to all examinations, including defence of thesis, at the Faculty of Medicine of Paris, to obtain his degree of medical doctor. He then went to serve his country in the torrid and humid Gabon on the west coast of Africa. On returning to France, he married and managed to be designated for a post where he could take with him his young wife: doctor on the Islands of Saint Pierre and Miquelon, a land of fishermen, with an extremely rigorous climate, situated south of Newfoundland in the North Sea. It was there that he began his self-taught study and practice of bacteriology, a new science at that time, carrying out a research which enabled him to be accepted by Roux in the course of Pasteur Institute and to become in the future one of the most illustrious pasteurians. After a training program of three months at the Institute, he was chosen by Pasteur himself to set-up and direct, in the then French colony of Indochina, a laboratory

for the preparation of vaccine which the incidence of 95% of smallpox in the native population made urgent, and also of the antirabic vaccine. In January of 1891, he leaved with his wife for the distant Cochinchina where, in Saigon he founded the Pasteur Institute's first branch. A fortuitous occurrence in October 1891 awakened his attention to the problem of snake-bite accidents and incited him to study snake venoms. A village in the vicinity of Bac-Lieu, about two hundred kilometers from Saigon was invaded by numerous cobras *(Naja naja)* fleeing from the inundation occurring there. The snakes entered the houses; forty natives were bitten and four died in few hours. An annamese, a combination of witch doctor and snake charmer, managed to capture nineteen of them which were sent to the recently created Bacteriological Institute of Saigon by the administrator of the region.

Fourteen arrived alive. Calmette sacrificed eleven in order to remove their venomous glands and obtain their venom. With the venom from the twenty two venomous glands he started in the laboratory at the Bacteriological Institute of Saigon his studies on snake venoms. A paper[14] was published in the "Annales de l'Institut Pasteur" in 1882 announcing favorable results, never confirmed, with the use of gold chloride in the treatment of animals injected with the venom. He stated in this paper that the repeated injection of warmed or unwarmed venom confers to the experimental animals a certain resistence to the venom, which was interpreted as some sort of mithridatism, not as a real state of immunity. Nonetheless, upon returning to Paris in 1893, he initiated at the laboratory of Roux in the Pasteur Institute, a research on the immunity conferred on the laboratory animals by the *Naja* and other snake venoms. The discovery by Behring and Kitasato[3] in 1890 of the antitoxic immunity in relation to diphtheria and tetanus toxins certainly stimulated the investigation. The results of this research were presented in February of 1894 to the *"Societé de Biologie"*[15] and also published in the same year in the *Annales de l'Institut Pasteur.*[16] His conclusions were correct, except one: the absence of specificity of the serum of the immunized animals. "Le sérum des animaux immunisés est *antitoxique, préventif et therapeutique* non seulement à l'egard du venin qui a servi à immuniser l'animal, mais même à l'egard des venins d'autres origines" he affirmed in his communication to the "Societé de Biologie" ("the serum from the immunized animals is preventively and therapeutically antitoxic not only in relation to the venom that was used to immunized the animal but even in relation to venoms of other origins"). In the work published in the "Annales de l'Institut Pasteur": "le sérum d'un lapin immunisé contre le venin de cobra ou de vipère agit indifferement sur tous les venins que j'ai experimentés" ("The serum from a rabbit immunized against the cobra or viper venom neutralizes as well all the venoms I have assayed"). The immunization of two mules was attempted at the Pasteur Institute where at the annex of Garches, thanks to a popular subscription, adequated installations were constructed in this period for Roux to initiate the production of the antidiphtheric serum, avidly required by the medical class of France and other countries for the treatment of croup. Production of the "sérum antivenimeux" took place in the city of Lille, where on the recommendation of Pasteur and Roux, Calmette set-up and directed for many years, the Pasteur Institute of Lille, a laboratory designated to attend the region of North France. Calmette never stated clearly which venoms he

used in the immunization of the horses.* It is certain, however, that the main venom if not the only one (since he did not admit the specificity of the antivenins) was that of *Naja naja* he received from Indochina. In subsequent years, several researches showed that Calmette's "sérum antivenimeux", although neutralizing the *Naja naja* venom, was incapable of doing so in relation of the venoms of other snakes, even of those pertaining to the Elapid family to which the *Naja naja* belongs. Martin,[24] professor of Physiology at the University of Melbourne, later on director of Lister Institute in London was the first or one of the first to disagree, in 1897, with Calmette's statement that a hyperimmune serum in relation to cobra venom is able to neutralize the venom of other snakes: "This statement (affirmation that the hyperimmune serum against *Naja naja* venom is equally efficient in neutralizing other snakes venoms) was surprising because Behring, from the examination of the relations of various toxins and antitoxins, had arrived at the conclusion that the curative value of immunising serum was specific, i.e., distinct toxins require distinct antitoxins, and some actions of different kinds of snake venom are quite as different as, say, the actions of the toxins of tetanus and yellow fever". He formulated, however, the hypothesis, also erroneous, that the specificity of antivenomous sera is due to the presence in snakes venoms of two types of proteic constituints, one being destroyed by heating to 75-85°C, of heavier molecular weight, predominant in the venom of the Viperidae, the other being resistent to the heating at these temperatures, of lighter molecular weight, predominant in the venom of the Elapidae. However, he did not immunize animals with these venom constituints in order to verify the correctness of his hypothesis. It was up to Vital Brazil to demonstrate the specificity of the antivenins by immunizing animals separately with the venom of different snake species and verifying that the sera from these animals neutralized exclusively or with much greater efficiency the venom which was used for the immunization. He was also the first to prepare mono and polyvalent antivenins for use in a determined region.

Vital Brazil was born in 1865, two years, therefore, after Calmette, in the little old town of Campanha on the highlands of the Mantiqueira, south of Minas Gerais. His high-school studies were made in São Paulo where his family settled residence when he was thirteen year old. Thereafter, he joined the Faculty of Medicine of Rio de Janeiro, one of the only two that operated in Brazil in the last century. While a student of medicine, he intended to study the venom of Brazilian snakes. He had to give up the idea: he did not meet the least receptivity to it on the part of the professors who had laboratories in the Faculty in which the research might be made. In Brazil at that time and unfortunately for still many years, the Faculties were considered to be only places of professionalizing education, never of research.

In 1881, at the age of 26, Vital Brazil received the degree of medical doctor. He, then, returned to São Paulo and worked in the first year of after-graduation as a doctor in the Police Force of the State of São Paulo.

* "J'ai étudié à ce point de vue le Sérum de Lille tel qu'il est fourni par le commerce; mais on ne sait pas exactement quels son les venins utilisés par Calmette à la préparation de son cheval à Serum", ("I have studied from this point of view the Lille's Serum as it is presented at the commerce; but it is not known exactly which venoms are used by Calmette for immunizing the horses that furnish the Serum") (from a letter of Maurice Arthus to Vital Brazil dated of February 11, 1911).

Vital Brasil
(1865 – 1950)

Afterward he joined the Public Health Department of the States as a Sanitarian Inspector. At that time, public health in São Paulo had deteriorated due to the abrupt increase in its population caused by the arrival of European immigrants, mainly from Italy, who were more susceptible to the endemic diseases, specially yellow fever, prevalent in the State. Besides they brought some others unknown in the country such as cholera morbus, of which a few outbreaks occured in São Paulo. Vital Brazil fulfilled with extreme dedication and competence the various commissions for which he was designated: to combat yellow fever in the hinterland of the State and cholera morbus in the valley of Parayba. For this gratification, his conduct in the fulfillment of his duties were always exalted by his superiors in the Public Health Department. Already married, with a daughter and limited financial resources, he did not think it right to risk so much his life. Therefore, he left the Public Health Department to practice medicine in the town of Botucatu in the hinterland of São Paulo, then a pioneerland, being quite successful. The idea of studying snake venoms once again occurred to him, now suggested by the verification of the ineficience of the medical resources to treat patients bitten by venomous snakes. He planned to investigate whether the plants claimed by the people to be efficient in the treatment of snake bite accidents would show any curative effect on envenomed laboratory animals. The results from these experiments were always negative. Taking knowledge, then, of the studies of Calmette on snake venom immunity, he understood that the antivenomous serotherapy was the right way to solve the problem of snake bite accident treatment. He decided thus, to return to São Paulo and to join the Bacteriological Institute

where he could count on resources to carry out the research on Brazilian snake venoms and snake venom immunity.

The Bacteriological Institute,[18] founded in 1892, was the first laboratory of bacteriology and parasitology in Brazil dedicated to public health problems. At the request of the Government of the State of São Paulo, Pasteur indicated the French bacteriologist Felix Le Dantec to set-up and direct it. However, after only four months, Le Dantec resigned and returned to Europe. He was substituted by Adolpho Lutz, already working at the Bacteriological Institute, on the recommendation by Le Dantec himself. "He is a Brazilian capable of directing the laboratory", he affirmed. Adolpho Lutz, son of Swiss, was born in Rio de Janeiro in 1855. He was raised, however, in Europe where he received the degree of medical doctor at the University of Bern, Switzerland, in 1880. Lutz had a very solid medical-scientific background, mainly in the field of morphological sciences, specially parasitology, entomology, mycology, zoology and pathology. He was a very competent director of the Institute, studying and elucidating with his coworkers at the laboratory the etiology and distribution in the State of various endemic and epidemic diseases. Vital Brazil joined the Bacteriological Institute in 1897 as an assistant. Lutz not only gave his consent to Vital Brazil to carry out the research on snake venoms at the Institute but also his help in solving some problems in its execution as, for instance, the most reliable process of catching venomous snakes: "Tendo nós entrado para o Instituto Bacteriológico (1897)", he wrote in 1901,[5] "onde tivemos permissão para continuar nossas pesquisas, vimos esta e outras dificuldades removidas pelo nosso sábio mestre Dr. Adolpho Lutz, que imaginou diversos aparelhos apreensores (de serpentes). Dentre eles o que melhores resultados práticos deu, foi o que nós denominamos laço". ("having joined the Bacteriological Institute (1897), where I had the permission to continue the research, this and other difficulties were done away with by Dr. Adolpho Lutz who planned various apparatus of snake trap. Among them the one we denominated as "laço" (lasso) gave the best results"). Vital Brazil extracted the venom from the most common venomous snakes in the State - rattlesnake (*Crotalus durissus terrificus*), jararaca (*Bothrops jararaca*), urutu (*B. alternatus*), jararacussu (B. jararacussu) — by the process still in use at the Butantan Institute nowadays. The amount of venom obtained from the different snake species was determined as well as their lethal doses in pidgeons, rabbits, guinea-pigs and dogs. The signs and symptoms evoked by the venoms on the experimental animals were faithfully described as well as the macroscopic lesions found at the autopsy of the animals. For the first time, the signs and symptoms as well as the lesions evoked by the South American rattlesnake venom on one hand and by jararaca and urutu venoms on the other were shown to be quite different. Immunization experiments on dogs, goats, oxen and horses were carried out. It was found[6] that "o cão é animal muito resistente e facilmente imunizável" ("The dog is an animal very resistant to snake venom and easily immunized") whereas "o cabrito, o boi e o cavalo são muito mais sensíveis e só a custo de grande trabalho e paciência consegue-se levar qualquer destes animais a um estado de imunização capaz de fornecer soro bastante ativo" (the goat, the ox and the horse are much more sensitive to the venom and only at the expense of much skill and patience does one manage to bring any one of these

animals to a state of high immunity''). Vital Brazil also found at this time (1898) that the antevenins are specific: ''Tendo imunizado um certo número de cães contra o veneno de cascavel e outros contra o de jararaca'', he wrote in 1901,[6] ''conseguimos soros bastante ativos, tendo verificado que o soro do animal imunizado contra o veneno de jararaca nenhuma ação tinha em relação ao da cascavel, bem como o soro muito ativo contra o veneno crotálico mostrava-se muito fraco em relação ao veneno da jararaca'' (''Having immunized some dogs against rattlesnake venom and others against jararaca venom, I obtained quite active sera, having found that the serum from animals immunized against jararaca venom did not neutralize that of rattlesnake, just as the very potent serum against the rattlesnake venom was quite weak in neutralizing the jararaca venom''). In 1899 he had to interrupt temporarily the research. ''A mortandade de ratos em Santos e o aparecimento de casos mórbidos que, por sua sintomatologia, tornaram-se suspeitos de peste bubônica inspiraram a Diretoria Geral do Serviço Sanitário a acertada providência de destacar para Santos um dos ajudantes do Instituto Bacteriológico com o instrumental necessário para, na primeira oportunidade, colher material de estudo e proceder a pesquisas bacteriológicas'', he recorded in a report of December, 1889 on the outbreak of bubonic plague in Santos.[7] (''Rat mortality in Santos as well as the occurence of cases of an illness which by its symptomatology was suspected to be bubonic plague, suggested the Administration of the Public Health Departament to send one of the Bacteriological Institute's assistants to Santos in order to collect, in the first opportunity, material for study and to proceed with bacteriological investigations''). Vital Brazil was indicated by Lutz for this mission. ''No dia 9 de outubro partimos para Santos'', he reported ''levando um microscópio, meios de cultura, pipetas, tubos esterilizados, ferros para autopsia etc. Instalamos nosso gabinete de observação em um dos quartos do Hospital de Isolamento''. (''On October 9, I left for Santos taking a microscope, culture media, pipettes, sterilized tubes, autopsy instruments etc. I set-up the laboratory in one of the rooms of the Hospital for Infectious Diseases''). From the buboes and blood of patients, Vital Brazil isolated in pure culture, a coccusbacillus that inoculated in rats, reproduced the disease. It was thus confirmed that the disease was really bubonic plague. The outbreak of plague in Santos called the attention of the São Paulo State Government to the necessity of setting-up a laboratory for the preparation of the antiplague serum which at that time was prepared in the Institute Pasteur at Paris in insufficient quantities to attend the requirements of the State of São Paulo and other Brazilian States, in view of the appearance of this disease in the country. Vital Brazil was indicated to set-up and direct, at a farm near São Paulo, called Butantan, a laboratory for the preparation of the antiplague and eventually other therapeutic sera. This laboratory, initially an annex of the Bacteriological Institute, became independent of this institution in 1901 under the name of Serotherapeutic Institute. Vital Brazil was nomeated its Director.

In 1901, Vital Brazil published in the São Paulo Medical Journal his first papers on snake venoms, some parts of which have herein been quoted.[5,6] In December of the same year he gave a lecture at the School of Pharmacy of São Paulo entitled ''Snake venom envenomation and its treatment''.[8] In this lecture, he discussed the various resources and methods proposed for

the treatment of snake bite accidents. Among them, those intended to prevent the absorption of the venom from the site of bite or to destroy it locally by physical or chemical means, and those whose aim is to neutralized the venom already absorbed: the antivenomous serotherapy introduced by Calmette and based on the discovery by this scientist[15] and Physalix and Bertrand[25] in 1884 of snake venom immunity. He reported that the antivenins are specific since Calmette's "sérum antivenimeux" did not exert any neutralizing action whatsoever on the rattlesnake venom while the serum of animals immunized with this venom, called by him anticrotalic serum, neutralized it perfectly well. Moreover, Calmette's serum and anticrotalic serum exerted only a very weak neutralizing action on jararaca venom while the serum from animals immunized with this venom was very potent in neutralizing it. The preparation at Butantan of the anticrotalic serum and antibothropic serum, this last one obtained from animals immunized with *Bothrops jararaca* and *Bothrops alternatus* venoms, and antiophidic serum, obtained by the mixture of the other two, was announced. The first case of a snake bite accident treated with an antivenin produced at Butantan was reported. In 1903 Vital Brazil made a very successful communication to the Fifth Brazilian Congress of Medicine and Surgery held in Rio de Janeiro, on snake-bite accident serotherapy and the specificity of the antivenins. At the opportunity, experiments on pidgeons, rabbits and guinea-pigs showing the preventive and curative efficacy of the sera prepared at Butantan were made. In 1903, he also published a paper[9] in the Medical Journal of São Paulo in which all his extensive researches on snake venom immunology were condensed. In this work not only the speficity of the antivenins was thoroughly demonstrated but also the existence, in certain cases, of a paraspecific action, that is, a serum obtained by the immunization of animals with a snake venom, can also neutralize, although to a lesser degree, the venom of a zoological close species exhibiting the same pharmacological actions. Twenty-one observations of patients bitten by venomous snakes treated by anticrotalic, antibothropic or antiophidic sera are also presented in this paper.

At a time of slow communications, it was necessary to make available to the rural populations the antivenins prepared at Butantan in order they might be applied as soon as possible in patients bitten by venomous snakes, and, at the same time to obtain snakes for venom extraction. For this, a service of exchange of the antivenins for snakes was established by Vital Brazil. Butantan furnished the farmers with the already mentioned "laços" for catching the snakes without danger of one being bitten and wooden containers for their transport to the Institute. Thanks to this service, thousands of venomous and nonvenomous snakes were received annually by Butantan, stimulating herpetological studies, initiated by Vital Brazil himself, set-up by his follower João Florencio Gomes and continued by Afranio do Amaral, Alcides Prado and more recently Alphonse Hoge.

In 1903, three years after the demonstration of antivenin speficity by Vital Brazil and the start of anticrotalic, antibothropic and antiophidic serum preparation at Butantan, George Lemb and William Hanna, from the Medical and Sanitary Department of Indian Government, published[22] their first paper on the specificity of the antivenins. They showed in this research that Calmette's "sérum antivenimeux" did not neutralize the venom from

the elapid *Bungarus fasciatus* nor those from the viperines *Echis carinatus* and *Vipera russellii*, snakes also responsable for accidents in India. "The outcome "they wrote", of all these observations is to prove conclusively that while the serum prepared by Calmette at Lille is of considerable value as a therapeutic measure in cases of cobra bite if injected sufficiently early and in sufficient quantity, it is of no value whatever in the treatment of cases of bites from *Daboia russellii, Bungarus fasciatus* or *Echis carinatus.*" They concluded: "these results... show conclusively that the serum prepared with a single venom would be specific for the venom of that species, that is to say inactive for poisons of other species of other genera". They published in a second article[23] coming out in 1904, the results of a research on antivenin specificity using a monovalent serum they prepared with the venom of cobra. The results of the previous study with Calmette's serum and a monovalent one obtained by the immunization of horses with tiger snake *(Notechis scutatus)* venom from Australia by Tidswell were confirmed. Besides, they showed that their anticobra venom serum only partially neutralized the venom of king cobra *(Ophiophagus hannah)* at that time classified in the genus *Naja (N. bungarus)* and whose venom is pharmacologically very similar to that of the commom cobra. "There is no doubt therefore that C.V. serum has a certain hindering effect on the action of king cobra venom *in vivo*. It cannot, however, be said to have a complete neutralizing effect even when used in large quantities. Further, it is certain that for pratical therapeutic purposes it would be of no value in cases of bites from this snake", they stated. Therefore, Lamb and Hanna's researches confirm entirely those of Vital Brazil on the specificity of the antivenins. Nowadays mono and polyvalent sera against the main snake venoms of India are prepared at Kasauli and Bombay.[20]

Frank Tidswell, from Australia, initiated his studies on snakes venoms of his country at the end of the last century. In 1902 he reported that the serum of horses hyperimmunized with the venom of tiger snake did not neutralize the venom from other Australian snakes. In 1906, an important contribution on Australian snake venoms, snake bite accidents and antivenin was published by Tidswell.[26] In it he emphasized: "The serum obtained (antivenin from horses hyperimmunized with the venom of tiger snake) could validly be regarded only as an antidote for tiger snake venom." He added: "Unfortunately it could act as an antidate only if the bite had been inflicted by a tiger snake". Curiosly enough, according to Chippaux and Goyffon,[20] only monovalent sera against the Australian snake venoms are produced in this country (possibly due to easy identification of the species of the snake causing the accident).

Notwithstanding the demonstration of the antivenin specificity by Vital Brazil, George Lamb and William Hanna as well as by Frank Tidswell, Calmette modified only partially, in 1907, his opinion on the subject. In his new concept only two components were responsible for snake venom toxicity and antivenin specificity: a neurotoxin predominant in venoms from the Elapidae and a hemorrhagine or proteolytic enzyme in those from the Viperidae.

In consequence he stated:[17] "Done, chez toutes les espèces de reptiles venimeux et peut-etre aussi chez d'autres animaux venimeux (tels que les scorpions), il semble que la substance *neurotoxique* soit *une* et toujour neutrali-

zable par un sérum *antineurotoxique* comme celui des animaux vaccinés contre le venin de *Cobra*." ("Therefore, in all species of venomous reptiles and perhaps in other venomous animals also (such as scorpions), it seems that there is but *one neurotoxic* substance which is always neutralizable by an *antineurotoxic* serum like that from animals immunized against *Cobra* venom"). Based on his belief in the unicity of snake neurotoxins he added: "Il" (the serum from animals immunized with cobra venom) "se montre de même trés suffisamment efficace á l'égard des venins de Colubridae* et de Viperidae dont l'activité neurotoxique peut entraîner la mort" ("It shows itself very sufficiently effective also in relation to the Colubridae* and Viperidae venoms whose neurotoxic action may cause death"). "Mais il ne possede action empechante", he affirmed further", sur les effects locaux de l'hemorragine à l'aquelle certains venins de viperidae tel les Lachesis** — doivent presque esclusivement leur nocuité" ("But it does not exert any hindering action on the local effects of the hemorrhagin to which some venoms of Viperidae — such as the Lachesis* — owe its noxiousness"). Vital Brazil in an article on antivenomous serotherapy published in 1909 dissented from Calmette's new ideas on venom specificity. "Infelizmente o grande número de experiências que temos realizado para elucidar esta questão", he wrote, "nos levam a discordar do ilustre professor não só com relação aos fatos em que se baseia como em relação às conclusões". (Unfortunately the large number of experiments I have done to elucidate this question, leads me to disagree with the eminent professor not only in relation to the facts on which he bases himself but also in relation to his conclusions**.) Showing a noteworthy fore-sight in view of the ignorance of venom chemistry at that time, he added: "A neurotoxina e a hemorragina são denominações puramente teóricas e não correspondem às substâncias isoladas e quimicamente puras. Indicam sintomas que se observam no decurso do envenenamento. O veneno de nossa cascavel (Crotalus terrificus) é neurotóxico, segundo a classificação do Professor Calmette, pois tem ação local muito limitada e mata por ação seletiva no sistema nervoso. A sua neurotoxina não pode, entretanto, ser identificada à do veneno de *Naja* não só pelas diferenças de propriedades como principalmente porque em doses imunizantes provoca formação de anticorpo diverso" ("The neurotoxin and the hemorrhagin are purely theoretical denominations and do not correspond to chemically pure isolated substances. They merely indicate symptoms that are observed in the course of the envenomation. The venom of our rattlesnake (*Crotalus terrificus)* is neurotoxic according to Professor Calmette's classification since it has a very limited local action, and kills by a selective action on the nervous system. Its neurotoxin cannot, ho-

* The elapids were then classified in two subfamilies (Elapinae and Hydrophiinae) of the Colubridae.
** Now *Bothrops*.

** Vital Brazil always demonstrated great respect and admiration for Calmette's scientific work and a high esteem for the great French scientist in spite of disagreing entirely with him in regard to the problem of antivenin specificity. Calmette on the other hand, always showed appreciation for Vital Brazil and his accomplishments. In 1928 by occasion of a homage rendered to Vital Brazil, he wrote: "L'oeuvre scientifique de Vital Brazil est de tout premier ordre. Ses travaux sur les venins et sur les serothérapies antivenimeuses ont sauvé des milliers d'existences. Je suis particulierement hereux de massocier à l'hommage que vous proposez de lui rendre, L'Institut Pasteur de Paris tout entier partage les sentiments de très haute estime et d'admiration que j'eprouve pour notre illustre collegue et ami".

wever, be identified with that of the *Naja* venom, not only because of differences of properties but principally because in immunizing doses, it gives rise to the formation of a distinct antibody.*

Maurice Arthus, the distinguished physiologist from Lausanne, decided in 1912, to reinvestigate the specificity of the antivenins in view of Calmette's affirmation in 1907 of the unicity of snake venom neurotoxins and hemorragins and because Pasteur Institute of Lille continued to prepare the "sérum antivenimeux" using exclusively or predominantly the *Naja naja* venom in the immunization of the horses. His objective: to elucidate whether venoms from distinct snake species exerting the same pharmacological actions are equally well neutralizable by an antivenin produced by one of them. In the first experiments[1], Arthus used the Lille "sérum antivenimeux" and the venoms from *Naja naja* (Cobra), *Ophiophagus hanna* (Hamadryas, King Cobra) and *Bungarus coeruleus* (Krait). After confirming that from the pharmacological point of view "les venins de Cobra, d'Hamadryas et de Krait sont rigoreusement equivalents" (the venoms of Cobra, Hamadryas and Krait are strictly equivalent"), he investigated the neutralizing activity of Calmette's serum in relation to these three venoms. He found that "pour neutraliser 1 mg de venin de Cobra, il faut 1.4 cc de sérum antivenimeux; pour neutraliser 1 mg de venin d' Hamadryas il faut environ 20 cc, soit 15 fois plus" (to neutralize 1 mg of Cobra's venom, 1.4 ml of the "sérum antivenimeux" are needed; to neutralize 1 mg of Hamadryas's venom, about 20ml of the sérum are needed or 15 times more"). Arthus further verified that Calmette's "sérum antivenimeux" even in a dose of 20 ml is only capable of hindering the death of the rabbits injected with 2 mg of *Bungarus coeruleus* venom (2 mg of the krait venom used equalled in toxicity 1 mg of that of Cobra). "De ces experiences, il resulte évidemment que la substance curarisante des trois venins considerés, la neurotoxine de Calmette, n'est pas une comme le pretend cet auteur: elle varie selon son origine zoologique puisqu'elle n'est pas modifiée semblablement à doses egales par le sérum anticobraíque" ("From these experiments, it is evident that the curarizing substance, Calmette's neurotoxin, in the three venoms is not one, as it is claimed by this author, since it is not modified in the same way when applied in equal doses, by the anti-Cobra venom serum"). In a second group of experiment[2], Arthus used not only Calmette's "sérum antivenimeux" but also antibothropic and anticrotalic sera from Butantan (furnished by Vital Brazil on Arthus's request) and the venoms from *Naja naja, Bothrops jararaca, Crotalus durissus terrificus, C. adamanteus* and *Pseudechis porphyriacus*. From the results of both researches, he concluded: "L'action des sérums antivenimeux est essentiallement specifique, il s'agit ici de especificité d'origine, de specificité zoologique et non pas de specificité d'action toxique" ("The action of the antivenins is essentially especific; it is a especificity of origin, of zoological especificity and not especificity of toxic action"). He made, however, the following restriction to the especificity of the antivenins: "Toutefois, cette lois de especificité zoologique comporte quelques exceptions" ("Nonetheless, this law of zoological

* Cobra and South American rattlesnake venoms induce neuromuscular blockade. Their neurotoxins are, however, chemically and pharmacologically, completely different (see Lee, C.Y., Chemistry and pharmacology of polypeptide toxins in snake venom, Ann. Rev. Pharmacol., 12:265-281, 1972; Vital Brazil, O., Neurotoxins from South American rattle snake venom, J. Formosan Med. Assoc., 71:394-400, 1972; Bon, C. et al., Postsynaptic affects of crotoxin and of its isolated subunits, Eu J. Biochemen., 99:471-481, 1979.

especificity admits some exceptions") and exemplifying: "Le sérum anticobraique exerce une action neutralisante, très faible d'ailleurs, sur les venins d'Hamàdryas et de Krait, très semblables au venin de Cobra. Les sérums antibothropique et anticrotalique agissent sur le venin de *Crotalus adamanteus* pour en suprimer ou tout au moins pour em atténuer les effets dépresseurs, mais non pour en neutralizer les effets coagulants *in vitro*. Le sérum anticrotalique supprime les effets coagulants *in vivo* du venin de *Pseudechis porphyriacus*, mais il n'agit pas sur ses autre proprietés toxiques ("the anti-Cobra venom serum exerts a neutralizing action, although very weak, on the Hamadryas and krait venoms which are very similar to that of Cobra. The anticrotalic and antibothropic sera suppress or at least attenuate the depressant effects of *Crotalus adamanteus* venom but they do not neutralize its coagulant effect *in vitro*. The anticrotalic serum suppresses the coagulant effect *in vivo* of *Psudechis porphyriacus* venom, but does not neutralizes its other toxic properties"). At the end of his second paper[2], Arthus gave the directives for obtaining really efficient antivenins, directives already established and followed by Vital Brazil since 1901 in the preparation of the antivenins at Butantan: "Pour traiter sérotherapeutiquement les moursures des serpents venimeux il faut préparer des sérums en immunisant les chevaux a l'aide du venin dont on se prepose de combatre les effets chez l'homme et chez les animaux mordus" ("To treat venomous snake-bite serotherapeutically, it is necessary that the antivenin be prepared by immunizing the horses with the venom whose effects one wants to counteract in bitten human beings or in animals").

After carrying out the researches already here referred to, Vital Brazil kept up his investigations on venoms and antivenins at Butantan. In 1907 he published a paper on the evaluation of the antitoxic activity of antivenins[10]. The very precise method he developed for this purpose is still in use nowadays at the Butantan Institute and other Brazilian laboratories. It was also adopted by the Malbran Institute of Buenos Aires and, with modifications, in other foreign institutions. A very extensive investigation on the venom of nearly all Brazilian poison snakes was published by Vital Brazil and Rangel Pestana in 1909[12,13]. The mean quantities of venoms obtained in thousands of extractions as well as their lethal doses for various laboratory animals are reported as well as their coagulant, hemolytic and proteolytic activities in *in vitro* experiments. The signs and symptoms as well as the lesions they evoke in dogs were also related. All of Vital Brazil's researches were characterized by great exactitude. The eminent physiologist from Argentine, Bernardo Houssay in a paper published in 1923[21] affirmed: "Nous avons confirmé toujour les recherches si exactes de Vital Brazil" ("We have confirmed always the researches so exact of Vital Brazil"). The researches of Vital Brazil on venoms and antivenins I have here summarized and those of Carlos Chagas on the American trypanosomiasis (Chaga's disease) are undoubtly the more important ones done in South America in the first decades of the present century.

At the end of this address, I want to mention what the distinguished American pathologist Simon Flexner, director of the Rockefeller Institute for Medical Research wrote in 1929 on Vital Brazil scientific work:

"It gives me great pleasure to express to you and your committee the profund admiration which I have for the scientific work of Dr. Vital Brazil,

the founder of the Instituto Butantan in São Paulo. The entire world is indebted to Dr. Brazil for his fundamental researches on the venoms and antivenins, and the benefits accruing from the institute he has developed are felt not only widely in Brazil, but even in distant countries.

I beg to join Dr. Brazil's colleagues in congratulating him on his past splendid work and in wishing him many more years of fruitful achievement."

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AVALIAÇÃO "IN VIVO" DA VACINA BIVALENTE CONTRA A GRIPE, PRODUZIDA NO INSTITUTO BUTANTAN.

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RESUMO: Verificou-se o poder de proteção da vacina bivalente contra a gripe (IB) em camundongos previamente imunizados e posteriormente infectados com suspensão de vírus influenza adaptado em pulmão de camundongo. Foram usados dois grupos de camundongos, correspondentes às imunizações com vacina pura e diluída a 10^{-1} e infectados com duas cepas do vírus influenza A/SP/1/81 (H_1N_1) e A/SP/1/80 (H_3N_2).
A imunidade foi considerada satisfatória, pois resultou sobrevivência além de 60% (Reed-Muench) para os dois grupos de animais.
PALAVRAS-CHAVE: Vacina contra a gripe, teste de proteção.

INTRODUÇÃO

O problema da gripe é de grande interesse mundial devido a alta infecciosidade da doença e sua incapacidade de produzir imunidade permanente. Pelo fato de que, de uma determinada epidemia de gripe, ou ainda por sua principal complicação, a pneumonia, decorram elevados coeficientes de morbidade e mortalidade, é que a Organização Mundial da Saúde (OMS) vem desenvolvendo um programa de colaboração internacional para o controle da infecção. Assim, coleta informações epidemiológicas de toda a parte do mundo, para divulgá-las rapidamente; ainda promove e coordena pesquisas de laboratório, a fim de descobrir novas variantes de vírus potencialmente infectantes, fornecendo-as aos laboratórios produtores de vacinas.

A prevenção e o controle da gripe têm sido sustentados, principalmen-

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te, por vacinas de diferentes tipos de preparo, com nível de proteção de até 80%, quando utilizadas em casos de epidemias [9].

A verificação da potência da vacina contra a gripe, geralmente se faz através de testes "in vitro", porém é importante a avaliação "in vivo" da proteção e para este teste são utilizados camundongos, hamsters e furões[4,11,12,13]. A padronização da vacina contra a gripe, através de testes comparativos "in vitro" e "in vivo", é referida nas investigações de Mc Laren e colaboradores[5].

Estudos da adaptação do vírus influenza em pulmão ou cérebro de camundongos adultos ou recém-nascidos, tem colaborado não somente no controle de vacinas, como também para esclarecimentos da infecciosidade e da neurovirulência de algumas cepas variantes do vírus [1,6,10].

O presente trabalho procura avaliar "in vivo" o poder imunizante da vacina bivalente contra a gripe, composta das cepas A/SP/1/80 (H_3N_2) e A/SP/1/81 (H_1N_1) inativadas, produzida no Instituto Butantan e que normalmente tem sua potência avaliada pela prova de hemaglutinação.

MATERIAL E MÉTODOS

Vírus

Foram empregados na pesquisa, as cepas do vírus influenza, A/SP/1/80 (H_3N_2) e A/SP/1/81 (H_1N_1), que são mantidas rotineiramente em passagens sucessivas na cavidade alantóide de ovos embrionados de galinha, com dez dias de idade. Com o líquido alantóide colhido após 40 horas da inoculação, foi preparado o inóculo [2].

Vacina

A purificação e a concentração do vírus foi realizada em supercentrífuga Sharples, com 50.000 r.p.m., fluxo contínuo, à baixa temperatura (+ 2 a 5°C) [8]. O vírus purificado foi inativado pelo formol na proporção de 1:4000 e ressuspenso em solução tampão fosfato (PBS), de acordo com título hemaglutinante, de onde resultou a vacina.

Adaptação do vírus em pulmão de camundongo

Para adaptação do vírus foram utilizados dois grupos de 10 camundongos com peso de 17 a 20 g, para inoculação das cepas variantes A/SP/1/80 (H_3N_2) e A/SP/1/81 (H_1N_1) com título hemaglutinante de 128 unidades cada[1,10]. Recebeu cada animal, 0,2ml de inóculo, por via nasal sob leve anestesia com éter. Após cinco dias os camundongos foram reinfectados obedecendo a mesma técnica utilizada anteriormente.

Decorrido o prazo de 10 dias, desde o início da primeira infecção, os pulmões dos animais inoculados foram retirados, triturados em gral e ressuspensos em solução tampão fosfato (PBS), usando-se 1ml para cada pulmão. Em seguida, o material foi centrifugado a 2000 r.p.m. por 30 min. e o sobrenadante foi testado quanto à infecciosidade, em um total de 20 camundongos para cada cepa do vírus, nas diluições 10^{-1} a 10^{-4}, com determinação do título infeccioso (DL_{50}), calculado pelo método Reed-Muench [6], no final do 10.º dia de inoculação. As cepas virais assim adaptadas foram utilizadas na prova de proteção da vacina.

Prova de Proteção "in vivo"

A prova de proteção da vacina, foi realizada em camundongos com peso de 17 a 20 gramas, inoculando-se por via intradérmica, 0,1ml da vacina bivalente contra a gripe, de título hemaglutinante de 400 unidades, composta das duas cepas virais em questão. Utilizou-se vinte camundongos para cada grupo, isto é, vacina pura e diluída 10^{-1}. Após sete dias, inoculou-se a segunda dose. Decorridos 15 dias após a primeira vacinação, os camundongos foram infectados com as cepas virais adaptadas, sendo que dez camundongos imunizados com a vacina pura foram infectados com 100 DL_{50} da cepa A/SP/1/80 (H_3N_2) e os outros dez, com 100 DL_{50} da cepa A/SP/1/81 (H_1N_1). Os animais imunizados com a vacina diluída a 10^{-1}, foram igualmente infectados e todos permaneceram em observação durante 25 dias. Um controle de 20 camundongos não-imunizados, porém infectados, foi incluído [5,13].

RESULTADOS

Os resultados obtidos estão resumidos na tabela.

Verificou-se, através do método de Reed-Muench, a sobrevivência acima de 50% para os dois grupos de camundongos e que coincidentemente apresentaram os mesmos resultados, tanto para vacina pura, como diluída (10^{-1}). Sendo 75% de proteção conferida pela vacina pura, contra 100 DL_{50} da cepa A/SP/1/80 (H_3N_2) e a mesma porcentagem de proteção, contra 100 DL_{50} da cepa A/SP/1/81 (H_1N_1). A vacina diluída a 10^{-1}, conferiu proteção de 60%, contra as mesmas DL_{50} das referidas cepas do vírus. No grupo controle dos camundongos infectados, mas não imunizados, os níveis de letalidade foram de 75% para a cepa A/SP/1/81 (H_1N_1) e de 65% para a cepa A/SP/1/80 (H_3N_2), o que foi considerado alta infectividade dos vírus empregados na prova de proteção.

CONCLUSÃO

Pelos resultados obtidos neste trabalho, parece-nos lícito concluir que a imunização dos camundongos, com vacina bivalente contra a gripe, foi satisfatória para o primeiro grupo de camudongos que recebeu vacina pura, contra 100 DL_{50} da cepa do vírus influenza A/SP/1/80 (H_3N_2) e 100 DL_{50} da cepa A/SP/1/81 (H_1N_1), pois apresentou sobrevivência acima de 50%. A imunização foi considerada satisfatória, quando este mesmo resultado foi obtido com o segundo grupo, imunizado com vacina diluída (10^{-1}) e contra as mesmas DL_{50} dos vírus influenza.

Podemos concluir ainda, que o teste de proteção "in vivo" é muito sensível, e importante no processo de avaliação da vacina contra a gripe, além dos testes "in vitro", tendo em vista que estes nem sempre estão associados ao poder imunizante, como observou Lacorte [2].

TABELA

Teste de proteção "in vivo" da vacina bivalente contra gripe

após infecção com vírus influenza

camundongos	N.º	% de Sobrevivência A (H_1N_1)	A (H_3N_2)
Vacinados	Vac. pura 20	75	75
	Vac. 10^{-1} 20	60	60
Não Vacinados (controle)	20	% de Letalidadc A (H_1N_1) 75	A (H_3N_2) 65

ABSTRACT: The protection of Influenza vaccine was verified in mice which had first been immunised and lateron infected with influenza virus adapted in mice's lungs. Two groups of mice were used corresponding immunizations with pure and diluted at 10^{-1} vaccine and two strains of influenza virus A/SP/1/81 (H_1N_1) and A/SP/1/80 (H_3N_2). The immunization was considered satisfatory, because of a survival of above 60% (Reed Muench) for the two groups of animals.
KEYWORDS: Vaccine against influenza, protection test.

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SPIDER VENOMS ACTING ON THE SODIUM CHANNEL *

Oswaldo VITAL BRAZIL **

ABSTRACT: Several toxins — animal, plant and microbial toxins act on the sodium channel. They may block it, slow down its inactivation or produce a persistent activation of it. The venom of spiders of the suborder Labidognatha, Ctenidae family, genus *Phoneutria* and of the suborder Orthognata, Dipluridae family, genus *Atrax* contain toxins that induce activation and/or slow down of the sodium channel. We have investigated the mechanism of action and the effects produced by *Phoneutria nigriventer* venom at the rat phrenic nerve-diaphragm muscle preparation. It was found that the venom caused a non-uniform depolarization of the diaphragm muscle fiber membrane which was abolished by tetrodotoxin or reduction of the sodium concentration in the bath fluid. The increase in the frequency of the miniature end-plate potentials induced by the venom was also suppressed by tetrodotoxin. On the other hand, the duration of action potentials was not increased by the venom. These results indicated that *Phoneutria* venom activates the voltage-dependent sodium channel in muscle and nerve cell membrane. All efects of venom on the phrenic nerve diaphragm muscle preparation can be explained on the basis of its action in the sodium channels. Sutherland studied the action of atraxotoxin, the main toxin from *Atrax robustus* venom, in the chicken *biventer cervicis* preparation. It was found that atraxotoxin induces spontaneous phasic contractions and enhances the response of the muscle to indirect stimulation. The spontaneous contractions were abolished by gallamine, succinylcholine, lowered calcium or elevated magnesium and by tetrodotoxin. This last observation suggests that atraxotoxin produces the spontaneous contractions by activating the sodium channel in nerve terminals.
KEYWORDS: *Phoneutria nigriventer* venom, *Atrax robustus* venom, sodium channel.

INTRODUCTION

The discovery of the mechanism of action of venoms and their toxins presents twofold interest. It permits to clear the pathophysiology of the en-

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venomations they produce and to improve their treatment, and/or to introduce in research new venoms or toxins that may become invaluable tools in physiological, pharmacological or pathophysiological investigations. The knowledge of the mechanism of action of such toxins as tetrodotoxin, saxitoxin, toxins from scorpions and some sea anemones, veratrum alkaloids, batrachotoxin and many others that act on the sodium channel has been of great usefullness from one or both points of view referred to. The study of the spider venoms acting on the sodium channel is only in its beginning. *Phoneutria nigriventer* venom activates the sodium channel in muscle and motor nerves [7,8] and may also slow down its inactivation in spinal nerve roots [3]. Sutherland experiments suggest that the main toxin from *Atrax robustus*, an Australian Dipluridae spider, activates also the sodium channel. Venoms of many other spiders from the Ctenidae or Dipluridae families probably act likewise activating and/or slowing down the sodium channel. They evoke signs and symptoms in experimental animals similar to those produced by *Ph. nigriventer* venom [13].

Phoneutria nigriventer venom

Ph. nigriventer (Ctenidae, Labidognatha), an aggressive wandering solitary spider from South America is responsible for most accidents of araneism in center eastern and southern Brazil. Its neurotoxic venom is very potent (Table I). The signs and symptoms it evokes in experimental animals or observed in human accidents are excruciating pain irradiating from the site of introduction, cramps, tremors, tonic convulsions, paralysis, salivation, diarrhea, sudoresis, priapism, tachycardia, arrhythmias and visual disturbances [4,5,10]. It does not produce local edema or necrosis, nor blood coagulation or hemolysis. The venom toxic components are polypeptides having molecular weight between 4000 and 6000 daltons [10,6].

TABLE 1

50% Lethal Dose to Mice of Some Arthropod Venoms Which Acts in the Sodium Channel

ARTHROPOD	Route of injection	LD 50 mg/kg
Leiurus quinquestriatus	subc.	0.25[a]
Androctonus australis	subc.	0.32[a]
Phoneutria nigriventer	subc. i.v.	0.67[b] 0.38[c]
Tityus serrulatus	i.v.	0.66[d]
Buthacus occitanus	subc.	0.90[a]
Centruroides sculpturatus	subc.	1.12[a]

a. Zlotkin et al., 1978.
b. Bucherl, 1983.
c. Fontana and Vital Brazil, 1985.
d. Vital Brazil et al., 1973.

We have investigated the mode of action of *Ph. nigriventer* venom at the isolated rat phrenic nerve-diaphragm preparation [14,7,8]. At the concentration of 5 ug/ml, the venom induced a tonic contraction with superimposed small phasic contractions in unstimulated diaphragms, both effects being suppressed by d-tubocurarine (Figure 1. I). Therefore, these effects must be ascribed to a presynaptic action of the venom producing acetylcholine release. In indirect stimulated diaphragms, the venom at concentrations of 1.0, 5.0 and 25.0 ug/ml produced the following effects: 1st.) a dose dependent tonic contraction of short duraction; 2nd.) small spontaneous phasic contractions, specially evident at venom concentrations of 5 ug/ml; 3rd.) an increase in twitch tension and delay in twitch relaxation; 4th.) a dose-dependent blockade of neuromuscular transmission at venom concentrations of 5.0 and 25.0 ug/ml, an effect partially antagonized by calcium but not by neostigmine or 4-aminopyridine (Figure 1, II, III and IV).

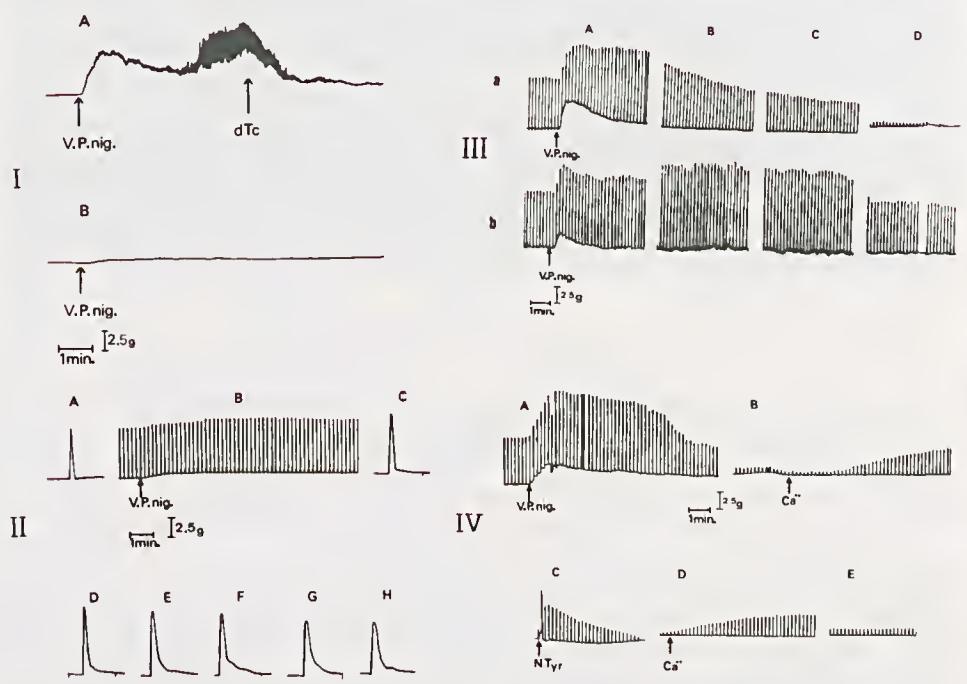

FIGURE 1 — Effects of *Phoneutria nigriventer* venom on muscle contraction. I. Unstimulated rat diaphragm. A, Addition of the venom, 5 ug/ml, and 14.6 uM d-tubocurarine to the bath. B, Addition of the venom, 5 ug/ml, to the bath containing a d-tubocurarine-treated rat diaphragm. II, III and IV, Indirectly stimulated rat diaphragms with maximal shock of 0.1 Hz and 0.2 ms. II, Effects of 1 ug/ml of the venom. A, Before venom addition to the bath; C, 10; D, 20; E, 70; F, 100; G, 140; H, 170 min after venom addition to the bath (paper speed: A, C, D, E, F, G, 5 cm/s; B. 0.02 cm/s). III, Effect of 25 (a) and 5 ug/ml (b) venom. B, C and D, 10, 20 and 40 min after venom addition to the bath. IV, Effect of calcium on venom-induced neuromuscular blockade. A, Addition of 25 ug/ml venom to the bath; B, addition of 10 mM $CaCl_2$; C, wash of the preparation with Tyrode solution; D, addition of 10 mM $CaCl_2$; E, 50 min after D. (Fontana & Vital Brazil, 1985).

In curarised directly stimulated diaphragm, the small phasic contractions did not occur. Therefore, they are due entirely to acetylcholine relea-

se. The tonic initial contraction only appeared in the d-tubocurarine treated diaphragms with the use of 25.0 ug/ml of venom. However, it was significantly smaller than that occuring in non-curarized preparations. The increase in twitch tension was smaller in directly stimulated diaphragms than in the indirectly stimulated ones when 1 and 5 ug/ml of venom were employed (Table 2). Twitch relaxation time was smaller in the curarized directly stimulated diaphragms. In summary, it can be said that at lower concentrations, *Phoneutria* venom presynaptic action is more important than the postsynaptic action in the genesis of the effects evoked at the rat phrenic nerve-diaphragm preparation.

TABLE 2

Effect of Phoneutria Nigriventer Venom on Twitch Tension

Data are reported as the increase in tension (grams) from the baseline to the peak of the twitches of rat phrenic nerve-diaphragm preparations. The directly stimulated preparations were blocked with 14.6 uM d-tubocurarine.

Venom (ug/ml)	Indirectly stimulated rat diaphragms		Directly stimulated rat diaphragms	
	Before venom	After venom	Before venom	After venom
1	7.3 ± 0.03	11.5 ± 0.08	7.0 ± 0.04	7.5 ± 0.07
5	8.0 ± 0.03	13.5 ± 0.07	8.7 ± 0.03	10.5 ± 0.06
25	8.0 ± 0.03	13.5 ± 0.06	8.0 ± 0.03	12.6 ± 0.03

The effect of *Ph. nigriventer* venom on end-plate potentials (e.p.p.s) were studied in d-tubocurarine blocked preparations. After venom, a single shock applied to the phrenic nerve induced a burst of repetitive e.p.p.s. (Figure 2). This result shows that the venom causes repetitive firing in the nerve, originated probably in the pre-terminal part of nerve endings. Repetitive evoked or spontaneous muscle action potentials were also produced by the venom (Figure 3). Action potential duration was not increased. This shows that the venom at the concentrations used does not slow down sodium channel inativation. The great increase of miniature end plate potential (m.e.p.p.) frequency induced by the venom was prevented by tetrodotoxin if added to the bath before venom. When added to the bath after the venom had greatly increased the frequency of the m.e.p.p.s., tetrodotoxin decreaseds it to normal levels (Figure 4). This effect shows that the increase in m.e.p.p. frequency is due to depolarization of the membrane of nerve endings resulting from activation of the sodium channel. The effect of *Phoneutria* venon on the muscle resting membrane potential was studied in preparations blocked by either d-tubocurarine or α-bungarotoxin and in unblocked diaphragms. The results did not differ significantly. Depolarization in five distinct regions of the diaphragm was investigated. It was found that *Ph. nigriventer* venom induced an unequal depolarization of the diaphragm muscle fiber membrane that was blocked by tetrodotoxin. End-plate and adjacent regions (R2 and R1, Figure 5) were much more depolarized by the venom than extrajunction regions (R4 and R5, Figure 5) of the diaphragm.

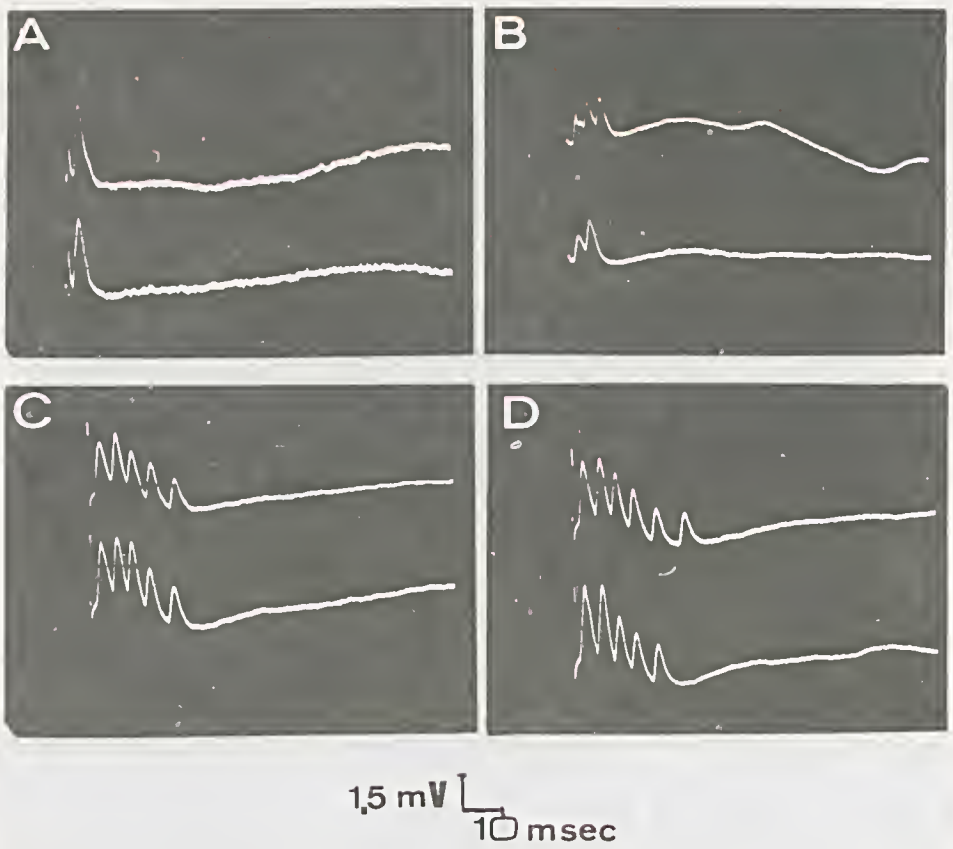

FIGURE 2 – Effect of *Phoneutria nigriventer* venom on the end-plate potential. The rat phrenic nerve-diaphragm muscle preparation was blocked with 0.73 uM d-tubocurarine. Nerve stimulation with single shocks. A, Control; B, C and D, 25, 30 and 40 min after addition of 5 ug/ml venom to the bath (Fontana & Vital Brazil, 1985).

In low sodium (17.2 mM) Tyrode solution the venom did not evoke depolarization. These results demonstrate that *Ph. nigriventer* venom activates the muscle sodium channel. A similar unequal depolarization of the diaphragm muscle fiber membrane is produced by veratrine [15,16] and crotamine [15], which activate also the sodium channel. This phenomenon may be due to a greater density of sodium channels at the end-plate region of the diaphragm or to a non-uniform distribution of activatable sodium channels by these toxins along the membrane of the diaphragm muscle fibers. The first hypothesis is favored by the findings that the maximum rate of rise of the action potential is greater at the end-plate than at extrajunctional regions [9,12,2] and that sodium-current density determined with the use of the loose patch voltage-clamp technique is much higher at regions immediately adjacent to the end-plate than at regions away from it [1].

In conclusion we may say:

1st.) *Ph. nigriventer* venom activates the sodium channel in nerve and muscle cell membrane. Its action in the sodium channel accounts for the effects produced in the rat phrenic nerve-diaphragm muscle preparation

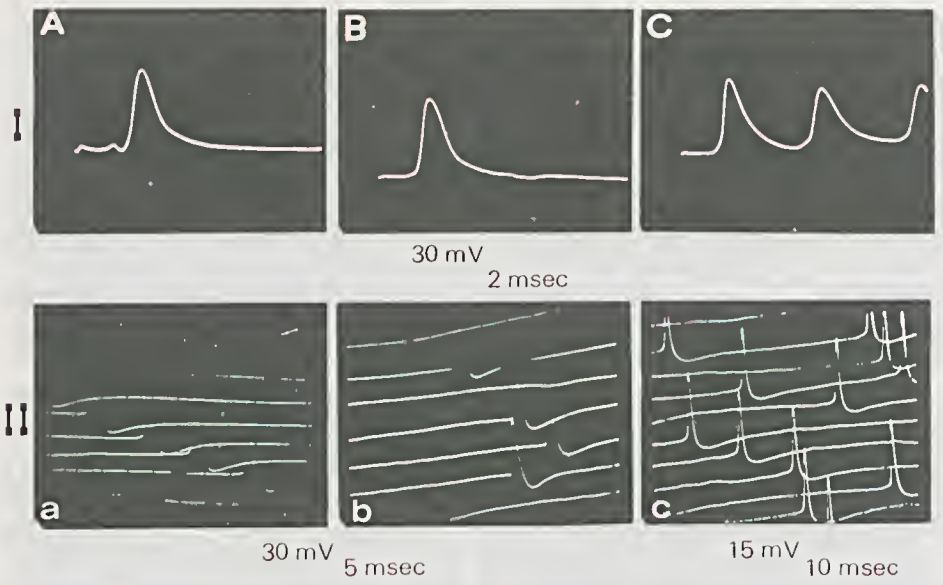

FIGURE 3 – Discharges of repetitive action potentials produced by *Phoneutria nigriventer* venom. I. Effect of 5 ug/ml venom on action potential evoked by nerve stimulation with a single shock II, Spontaneous discharges of action potentials in preparations treated with 1.0 (a), 5.0 (b) and 25.0 (c) ug/ml venom (Fontana & Vital Brazil, 1985).

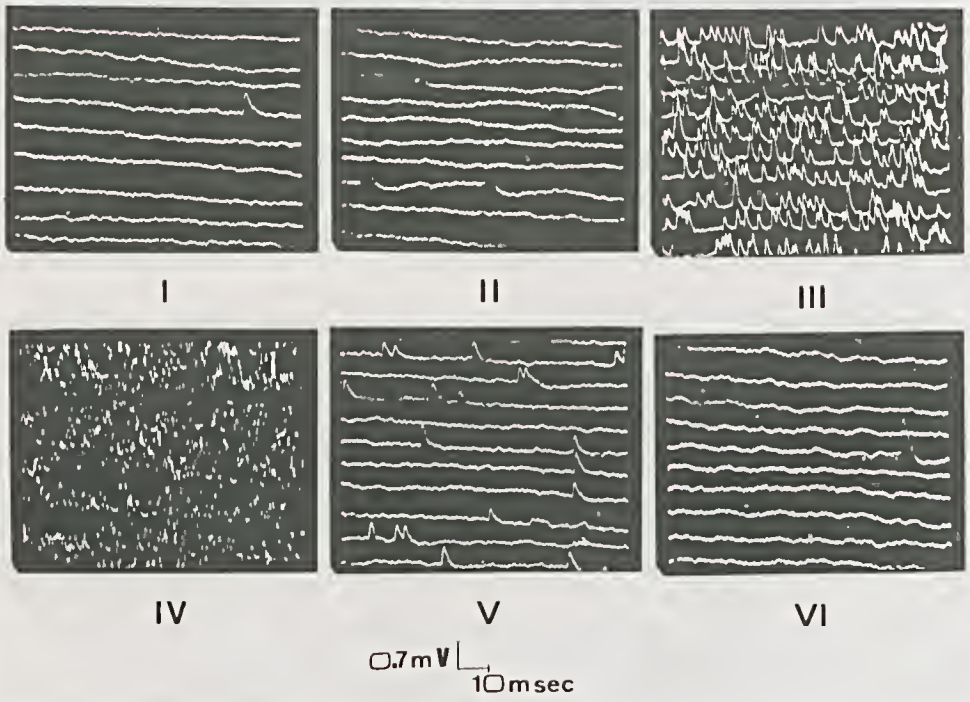

FIGURE 4 – Effect of *Phoneutria nigriventer* venom on the miniature end-plate potentials at the rat phrenic nerve-diaphragm muscle preparation. I, Control; II, III and IV, 10, 20 and 30 min after the addition of 5 ug/ml venom to the bath; V and VI, 5 and 15 min after the addition of 3 uM tetrodotoxin to the bath (Fontana & Vital Brazil, 1985).

and may be responsible for all signs and symptoms observed in experimental or clinical envenomation.

2nd.) *Ph. nigriventer* venom induces bursts of repetitive action potentials which may appear after an evoked action potential or spontaneously. Spontaneous action potential generation denotes the venom acts also reducing the threshold potential of the excitable cell membrane, that is, the threshold potential becomes more negative under the action of *Ph. nigriventer* venom.

3rd.) Neurotransmitter release produced by evoked or spontaneous bursts of repetitive action potentials is the main or unique cause of such effects as spontaneous phasic or tonic contractions, increase in twitch tension and delay in twitch relaxation.

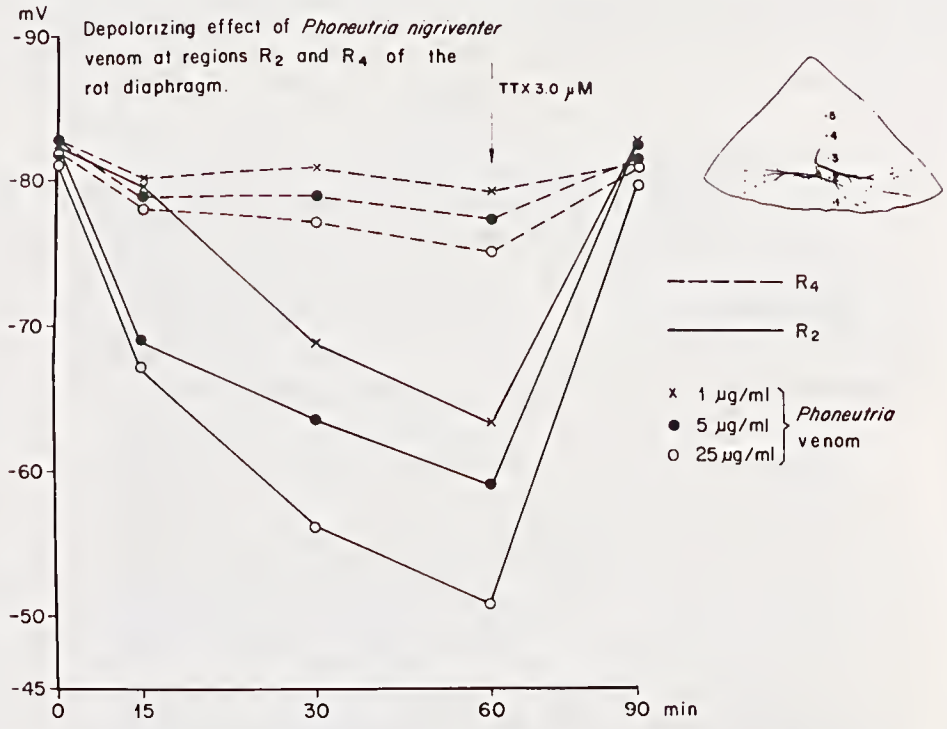

FIGURE 5 — Depolarization produced by *Phoneutria nigriventer* venom at the end-plate (R 2) and an extrajunctional region (R 4) of the rat diaphragm. The preparation was blocked with d-tubocurarine (14.6 uM). Three concentrations (1.0, 5.0 and 25 ug/ml) of venom were use. Tetrodotoxin (3.0 uM) was added to the bath 60 min after venom. Each point in the curves is the mean of three experiments.

Atrax robustus Venom

The Orthognatha spiders of the Dipluridae family, genus *Atrax* occur in southeastern Australia. *A. robustus*, a species from the central coastal region of New South Wales and Blue Mountain region to the west is responsible for severe human accidents. However, fatalities from them are very low. The signs and symptoms of envenomation are severe local pain lasting

for hours or even days, nausea and vomiting, abdominal pain, diarrhea, salivation, lacrimation, sweating, hypertension, dyspnea, local and generalized muscle fasciculations. Muscle twitching may be prolonged and violent. Hypotension in some patients preceeds cardiac arrest.

Sutherland [11] studied the action of atraxotoxin, the main toxin from *A. robustus* venom, in the chicken *biventer cervicis* preparation. He found that atraxotoxin induces spontaneous phasic contractions and enhances the response of the muscle to indirect stimulation. The spontaneous contractions were abolished by gallamine, succinylcholine, lowered calcium or elevated magnesium. These results show that the contractions were produced by acetylcholine release caused by discharge of action potentials in the nerve, very probably originated in the pre-terminal part of the nerve endings. The effect of tetrodotoxin abolishing the spontaneous contractions suggests but does not prove that atraxotoxin acts in the nerve sodium channel. Further experiments with the use of electrophysiological techniques are necessary to clarify if atraxotoxin activates or not the sodium channel.

RESUMO: Várias toxinas — de origem animal, vegetal ou microbiana — atuam no canal do sódio. Podem bloqueá-lo, retardar sua inativação ou produzir ativação persistente do mesmo. A peçonha de aranhas da subordem Labidognatha, família Ctenidae, gênero *Phoneutria* e da subordem Orthognatha, família Dipluridae, gênero *Atrax*, contém toxinas que induzem ativação e/ou retardo da inativação do canal do sódio. Investigamos o mecanismo de ação e os efeitos da peçonha de *Phoneutria nigriventer* na preparação nervo frênico-diafragma de rato. Verificamos que a peçonha causa despolarização desigual da membrana das fibras musculares de diafragma abolida quer pela tetrodotoxina quer pela redução da concentração em íons sódio na solução nutritiva. O aumento da freqüência dos potenciais da placa terminal em miniatura produzido pela peçonha também foi abolido pela tetrodotoxina. Por outro lado, a duração do potencial de ação, evocado ou espontâneo, não foi alterada pela peçonha. Estes resultados mostram que a peçonha de *Phoneutria* ativa o canal do sódio voltagem-dependente da membrana de fibras nervosas e musculares. Todos os efeitos produzidos pela peçonha na preparação nervo frênico-diafragma de rato podem ser explicados como decorrentes de sua ação no canal do sódio. Sutherland investigou a ação da atraxotoxina, a principal toxina da peçonha de *Atrax robustus*, na preparação *biventer cervicis* de pintainhos. Verificou que a atraxotoxina induz contrações fásicas espontâneas e aumenta as respostas do músculo à estimulação indireta. As contrações espontâneas foram abolidas pela galamina, pela succinilcolina, pela redução na concentração de cálcio ou elevação da de magnésio na solução nutritiva e pela tetrodotoxina. Esta última observação sugere que a atraxotoxina produza as contrações espontâneas ativando o canal do sódio nas terminações nervosas.

PALAVRAS-CHAVE: peçonha de Phoneutria nigriventer; peçonha de Atrax robustus, canal do sódio.

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